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(54) Title: HUMAN PLACENTA ANGIOGENIC FACTOR CAPABLE OF STIMULATING CAPILLARY ENDOTHELIAL CELL PROTEASE SYNTHESIS, DNA SYNTHESIS AND MIGRATION

(57) Abstract

An angiogenic factor is disclosed which is a purified, single-polypeptide-chain protein having at least one active site possessing an activity selected from the group consisting of mitogenic activity, chemotactic activity, the ability to stimulate protease synthesis and combinations thereof. This angiogenic factor exhibits substantial homology to and is immunologically equivalent to the native angiogenic factor isolatable from human placental tissues. The amino acid sequence of this angiogenic factor is also disclosed. In addition, a method for isolation of the purified angiogenic factor from human placental tissues is set forth. Pharmaceutical preparations incorporating this angiogenic factor are described.

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HUMAN PLACENTA ANGIOGENIC FACTOR CAPABLE OF STIMULATING CAPILLARY
ENDOTHELIAL CELL PROTEASE SYNTHESIS, DNA SYNTHESIS AND MIGRATION

This application is a continuing application of U.S. Serial
No. 809,873, filed December 17, 1985, of Moscatelli et al. enti-
tled Human Placenta Angiogenic Factor Capable of Stimulating
Capillary Endothelial Cell Protease Synthesis, DNA Synethesis
and Migration.

BACKGROUND OF THE INVENTION

Angiogenic factors have been defined as proteins which
possess a variety of properties, namely the ability to (1) in-
crease the rate of endothelial cell proliferation; (2) increase
endothelial cell protease synthesis; (3) stimulate endothelial
cell migration toward the protein location; and (4) cause in
vivo capillary proliferation. In particular, it has been ob-
served that substances classified as angiogenic factors can be
mitogenic by affecting DNA synthesis in endothelial cells, thus
increasing the rate of endothelial cell proliferation and the
rate at which new blood vessels are formed.

Interrelated with this property is the ability of
angiogenic factors to increase protease synthesis by endothelial
cells. These proteases include plasminogen activator (PA) and
collagenase. Specifically, the angiogenic factors are able to
stimulate synthesis of PA and latent collagenase where the PA
can convert zymogen plasmin into active plasmin, a protease of
wide specificity, which in turn can convert latent collagenase
into active collagenase. These two proteases, active plasmin
and active collagenase, are capable of degrading most of the
proteins in surrounding tissues, thus allowing increased inva-
sion of various tissues, such as capillary endothelial cells.
Moreover, angiogenic factors are chemotactic for certain cells,
particularly capillary endothelial cells, i.e. they induce these
cells to migrate toward the angiogenic factor.

With these properties in mind, it has been postulated
that the isolation of an angiogenic factor would allow creation
of a therapeutic substance capable of increasing the blood sup-
ply to an organ. For instance, subsequent to certain myocardial
infarctions it would be desirable to stimulate regeneration of
the blood supply to the heart interrupted as a result of the
infarction or to stimulate re-growth of vessels in chronic ob-
structions. In addition, the use of an angiogenic factor may

stimulate healing in decubitus ulcers, surgical incisions and
slowly healing wounds, particularly in geriatric and diabetic
patients. Moreover, the application of this material to burns
may improve the rate and degree of healing. Therefore, a
purified angiogenic factor suitable for therapeutic applications
in humans has been sought. Additionally, some scientists
believe that study of a substance capable of stimulating blood
vessel growth may lead to processes for which the blood supply
to a cancerous tumor might be inhibited, thus starving the can-
cer.

Previously, although a class of proteins had been
identified which have been referred to as "angiogenic factors,"
these proteins were primarily isolated from non-human sources.
It is believed that angiogenic factors isolated from non-human
sources would not be suitable for use as therapeutic agents in
humans due to the potential for adverse immunological reaction
in response to a foreign protein. Moreover, it had not been
demonstrated whether these non-human proteins individually pos-
sessed the four identified properties of an angiogenic factor
identified above or whether the observed properties were attrib-
utable to the interactions between a combination of proteins.

Indeed, various proteins which have been found to have
endothelial cell mitogenic properties have been divided into two
classes: endothelial cell growth factor-like molecules which
are eluted from heparin-Sepharose with 1 M NaCl and which have
an acidic pI; and fibroblast growth factor-like molecules which
bind more strongly to heparin-Sepharose and which have a basic
pI. In addition, the present inventors believe that there is a
third species of angiogenic factor, that described as
"angiogenin" in papers recently published by Vallee et al. of
Harvard Medical School, in Biochemistry, 1985, Vol. 24, pgs.
5480-5499. It is believed that angiogenin, while possessing
some properties of a true angiogenic factor, is a distinct spe-
cies in that it lacks mitogenic properties.

In the face of this patchwork of research, the present
inventors sought and discovered a human angiogenic factor,
classifiable as an FGF_{basic}, which is substantially homologous
to that isolatable from human placental tissue, which, in a

single molecule, has the above-identified properties, i.e., is mitogenic, chemotactic, and capable of stimulating protease synthesis as well as capable of causing _in vivo_ capillary proliferation. Furthermore, the present inventors sought to isolate this angiogenic factor in a substantially purified form from human placental tissues. The amino acid sequence of this isolated angiogenic factor has now been determined. It is believed that the determination of this amino acid sequence will allow identification of DNA probes for use in and obtaining genomic or cDNA sequences useful in recombinant-DNA methods for the synthesis of angiogenic factors.

SUMMARY OF THE INVENTION

The present invention relates to angiogenic factors generally, and more specifically, to those angiogenic factors classifiable as FGF_{basic}. In particularly, this invention relates to an FGF_{basic} angiogenic factor which is substantially equivalent to that isolatable from human placental tissues, and which has mitogenic and chemotactic properties and which is capable of inducing protease synthesis and, _in vivo_, causes capillary proliferation.

An object of the present invention is to provide purified forms of an angiogenic factor which possess these properties. An additional object of the present invention is the determination of the amino acid sequence of such an angiogenic factor. A further object of the present invention includes providing purified forms of FGF_{basic} which would be valuable as pharmaceutical preparations exhibiting mitogenic and chemotactic properties along with the ability to stimulate protease synthesis.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned from practice of the invention. These objects and advantages may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, an angiogenic factor is

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disclosed which has at least one active site possessing an
activity selected from the group consisting of mitogenic activ-
ity, chemotactic activity, the ability to stimulate protease
synthesis, and combinations thereof. The human or synthetic
angiogenic factor is classified as an FGF$_{basic}$ and exhibits sub-
stantial homology to the native angiogenic factor isolatable
from human placental tissue.

It should be noted that, while it is preferred that
the angiogenic factor itself be capable of stimulating protease
synthesis, the term "protease," as used herein, includes active
or precursor forms. Examples of such precursor forms include
latent or pro-collagenase. Moreover, it is possible that some
angiogenic factors may be isolated that are encompassed within
the scope of the present invention but which do not directly
stimulate protease synthesis. These angiogenic factors, how-
ever, do cause biological responses which in turn stimulate
protease synthesis. Thus, the angiogenic factors of the present
invention either directly or indirectly stimulate protease syn-
thesis.

A particularly preferred angiogenic factor according
to the present invention has the following core amino acid se-
quence:

    L-Y-C-K-N-G-G-F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-
    S-( )-P-H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-A-N-
    R-Y-L-A-M-K-( )-D-G-( )-L-L-A-( )-K-( )-V-T-( )-E-( )-F-
    F-F-E-( )-L-E-S-N-N-Y-N-T-Y-R-( )-

In addition, peptides having the sequences

    K-L-G-S-K-T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K and

    Y-( )-S-W-Y-V-( )-L-( )

are present in the polypeptide outside the core sequence. In
the sequences depicted herein, open parentheses indicate the
presence of a single amino acid residue that is not completely
identified. Another particularly preferred angiogenic factor
has the following sequence:

    G-T-M-A-A-G-S-I-T-T-L-P-A-L-P-E-D-G-G-S-
    G-A-F-P-P-G-H-F-K-D-P-K-R-L-Y-C-K-N-G-G-
    F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-S-D-P-
    H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-
    A-N-R-Y-L-A-M-K-E-D-G-R-L-L-A-S-K-C-V-T-

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D-E-C-F-F-F-E-R-L-E-S-N-N-Y-N-T-Y-R-S-R-
K-Y-T-S-W-Y-V-A-L-K-R-T-G-Q-Y-K-L-G-S-K-
T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K-S.
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The amino acids represented by the foregoing abbreviations are
set forth in the Description of the Preferred Embodiments below.

Futhermore, to achieve the objects and in accordance
with the present invention, a substantially purified form of the
native angiogenic factor isolatable from human placental tissue
is disclosed. Additionally, to achieve the objects and accor-
dance with the purposes of the present invention, pharmaceutical
compositions containing, as at least one of the active ingredi-
ents, an angiogenic factor in accordance with the present inven-
tion as set forth herein are disclosed.

It is to be understood that both the foregoing general
description and the following detailed description are exemplary
and explanatory only and are not restrictive of the invention,
as claimed.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

References will now be made in detail to the presently
preferred embodiments of the invention, which, together with the
following examples, serve to explain the principles of the
invention.

As noted above, the present invention relates to an
angiogenic factor which has been isolated in purified form.
Preferably, the angiogenic factor of the present invention is a
single-polypeptide-chain protein which is substantially homolo-
gous to, immunologically equivalent to, and, most preferably,
biologically equivalent to, native angiogenic factor isolatable
from human placental tissues. By "biologically equivalent," as
used throughout this specification and claims, it is meant that
the composition of the present invention possesses mitogenic and
chemotactic properties and is capable of inducing protease syn-
thesis in the same manner, but not necessarily to the same
degree, as the native angiogenic factor.

By "substantially homologous," as used throughout the
ensuing specification and claims, is meant a degree of homology
to the native angiogenic factor in excess of that displayed by
any previously reported, purified, substantially homologous

angigenic factor composition. Preferably, the degree of
homology is in excess of 50%, preferably 60%, and more prefer-
ably 75%, with particularly preferred proteins being in excess
85% or 90% homologous with the native protein. The degree of
homology as described above is calculated as the percentage of
amino acid residues found in the smaller of the two sequences
that align with identical amino acid residues in the sequences
being compared when four gaps in a length of 100 amino acids may
be introduced to assist in that alignment as set forth by
Dayhoff, M.O. in Atlas of Protein Sequences and Structure, Vol.
5, page 124 (1972), National Biochemical Research Foundation,
Washington, D.C., specifically incorporated herein by refer-
ences.

        As described herein, the angiogenic factor of the
present invention is either isolated from a human source or is a
synthetic polypeptide. The term "synthetic" polypeptide is in-
tended to mean an amino acid sequence which has not previously
been isolated from nature in a substantially purified form. In
applying this definition, "synthetic" encompasses, among others,
polypeptides created by recombinant-DNA methods or synthesized
in whole or in part in vitro. In particular, synthetic poly-
peptides are contemplated in which 1 or 2 amino acids differ
from those set forth in the preferred sequences set forth below.

        The preferred angiogenic factor of the present inven-
tion has been discovered in human placental tissue extracts and,
for the first time, has been isolated in a purified form. For
the purposes of the present application, "pure form" or
"purified form," when used to refer to the angiogenic factor
disclosed herein, shall mean substantially free of other pro-
teins which are not angiogenic factors. Preferably, the
angiogenic factor of the present invention is at least 50% pure,
more preferably 70% pure and even more preferably 80% or 90%
pure.

        Additionally, the angiogenic factor of the present
invention has been isolated from various tumor and normal cells.
These include SK-Hepl cells, HeLa cells and K562 cells, as well
as human embryonic lung fibroblasts.

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The angiogenic factor of the present invention may be
isolated in pure form from human placental tissues by the method
comprising: (a) collecting human placental tissues; (b)
isolating the angiogenic factor from the human placental tissues
by fractionating the proteinaceous material in the tissues; (c)
identifying the fractions which possess angiogenic factor activ-
ity; and (d) concentrating the fractions exhibiting the
angiogenic factor activity.

In a preferred embodiment, the proteinaceous material
present in the human placental tissues is fractionated using a
combination of heparin affinity chromatography, ion exchange
chromatography and, optionally, gel permeation chromatography.
The angiogenic factor discussed herein may also be isolated
through the use of monoclonal antibodies with a specificity for
the placental proteins. In this embodiment, antigen is bound to
a matrix (resin) containing monoclonal antibodies against the
placental protein and non-antigenic proteins are removed by
washing the resin with buffer. The antigen is then removed from
the antibody by the use of a buffer of either high or low pH, or
high ionic strength, or chaotropic agents, alone or in combina-
tion with a change in temperature.

Fractions thus obtained are screened for the presence
of angiogenic factor activity. Preferably, this is accomplished
in part by evaluating the effect on PA and collagenase synthesis
by incubating appropriate endothelial cell cultures, preferably
mammalian capillary endothelial cells, in the presence of the
angiogenic factor and assaying the medium for latent collagenase
and the cells for PA. The amount of protease produced by the
cells stimulated by the angiogenic factor may also be determined
by immunological methods such as ELISA or RIA assays or
immunoprecipitation methods.

The mitotic ability of the angiogenic factor is pref-
erably measured by incubating appropriate endothelial cells,
preferably mammalian capillary endothelial cells, in the pres-
ence of the angiogenic factor and a radiolabelled nucleotide,
preferably ^{125}I-iododeoxyuridine (^{125}I-dU). The amount of
^{125}I-dU incorporated into trichloroacetic acid insoluble mate-
rial is then measured as indicative of the extent of DNA

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synthesis. The chemotactic abilities of an angiogenic factor
are preferably demonstrated by incubating an appropriate
endothelial cell culture, preferably mammalian capillary
endothelial cells, in the presence of the angiogenic factor and
measuring cell motility in an appropriate vessel, preferably a
modified Boyden chamber.

        As noted above, the present inventors have succeeded
in isolating an angiogenic factor from human placental tissues
in a hitherto unavailable, purified form. Isolation of this
protein in a purified form was a prerequisite step to the cor-
rect sequencing of the protein and to the development of pharma-
ceutical compositions containing the angiogenic factor.

        A preferred angiogenic factor of the present invention
has the following core amino acid sequence:

        L-Y-C-K-N-G-G-F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-
        S-()-P-H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-A-N-
        R-Y-L-A-M-K-()-D-G-()-L-L-A-()-K-()-V-T-()-E-()-F-
        F-F-E-()-L-E-S-N-N-Y-N-T-Y-R-()-

In addition, peptides having the sequences

        K-L-G-S-K-T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K and

        Y-()-S-W-Y-V-()-L-() .

are present outside the core sequence. Another particularly
preferred angiogenic factor has the following sequence:

        G-T-M-A-A-G-S-I-T-T-L-P-A-L-P-E-D-G-G-S-

        G-A-F-P-P-G-H-F-K-D-P-K-R-L-Y-C-K-N-G-G-

        F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-S-D-P-

        H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-

        A-N-R-Y-L-A-M-K-E-D-G-R-L-L-A-S-K-C-V-T-

        D-E-C-F-F-F-E-R-L-E-S-N-N-Y-N-T-Y-R-S-R-

        K-Y-T-S-W-Y-V-A-L-K-R-T-G-Q-Y-K-L-G-S-K-

        T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K-S.

The foregoing abbreviations correspond to the standard abbrevia-
tions for amino acid residues as set forth in, for example,
Biochemistry by A.L. Lehninger, 2nd ed., Worth Publishers, Inc.,
New York (1975), pg. 72.

        It is believed that the activity of the claimed
angiogenic factors is not affected if any or all of the fifteen,
sixteen, seventeen, or eighteen N-terminal amino acid residues
are removed from the intact polypeptide. Thus, it is intended

that all of these abbreviated sequences are encompassed in the present invention. Moreover, the extension of the amino acid sequence by the addition of up to 110 amino acids to the N-terminal amino acid of the intact polypeptide are also contemplated.

It is also contemplated that additions of polypeptide chains to the C- or N- terminus of the present angiogenic factor will be within the scope of the present invention. In particular, polypeptide chains may be joined to either terminus through protein fusion techniques. These additional polypeptides may serve to enhance the pharmacological efficacy of the instant angiogenic factors. For example, the polypeptide may, by fusion with other polypeptides, be made more capable of retaining its activity in the presence of low pH or high temperature, or the resultant polypeptide may possess a longer circulating life, greater resistance to degradation or increased ability to be transported across the intestinal epithelia.

However, it should be noted that, in these alterations, the variation to the amino acid sequences should not be such as to provoke an adverse immunological response in the organism to which the angiogenic factor is administered where such adverse response would be determined to be of such detriment to the organism that the benefits derived from the angiogenic factor would not be warranted. The methods of determining whether a biological molecule would provoke such an adverse immunological response are known to those of ordinary skill in the art.

The angiogenic factor of the present invention and its analogs as disclosed herein are contemplated for human and veterinary uses in the form of pharmaceutical products possessing mitogenic or chemotactic properties or having the ability to stimulate protease synthesis. It is expected that pharmaceutical preparations containing, as at least one of active ingredients, one of the present angiogenic factors would also contain appropriate, pharmaceutically acceptable carriers, diluents, fillers, binders and other excipients depending on the dosage form contemplated. For oral administration, steps must be taken to prevent degradation of the active protein in the digestive

tract. Enteric coated dosage forms are thus contemplated as one
form suitable for oral administration. If parenteral adminis-
tration is chosen, the preparation may contain a water or saline
solution or other pharmaceutically acceptable suspension agent.
Generally, it would be preferred that a preparation intended for
parenteral administration contain sodium chloride in sufficient
concentrations to make the overall preparations isotonic to body
fluids. It is also contemplated that the pharmaceutical prepa-
rations containing the angiogenic factor of the present inven-
tion be administered locally, as by injection or topical appli-
cation, for treatment of wounds, surgical incisions or skin
ulcers. Additionally, incorporation of the angiogenic factor
into a slow release implant device is contemplated for adminis-
tration to regenerate the blood supply to the heart after a
myocardial infarction.

The calculations necessary to determine the appropri-
ate dosage for treatment of each of the above-mentioned disor-
ders and appropriate for use with the described delivery methods
are routinely made by those of ordinary skill in the art and are
within the ambit of tasks routinely performed by them without
undue experimentation, especially in light of standard assays
and the assays disclosed herein. These dosages may be ascer-
tained through use of established assays for determining dosages
utilized in conjunction with appropriate dose-response data.

It is understood that the application of the teachings
of the present invention to a specific problem or environment
will be within the capabilities of one having ordinary skill in
the art in light of the teachings contained herein. Examples of
the products of the present invention and representative pro-
cesses for their isolation and manufacture appear in the follow-
ing examples.

Example 1

Purification of a Human Angiogenic Factor from
Placenta Tissues.

A.   Protein Purification

Term human placentas were frozen at -20°C after
delivery. The frozen placentas were broken into small pieces,
ground with an electric food chopper (General Slicing, Walden,

NY), and homogenized in a food processor. After homogenization, all subsequent steps were performed at 4°C. The homogenized placentas were diluted with cold 20 mM Tris, pH 7.5, 3 mM EDTA and were sonicated for 10 min. at 50 W (model 185 sonicator, Branson Sonic Power Co., Plainview, NY). Generally, 1 kg of frozen placenta yielded 2 liters of sonicate.

The sonicate was brought to pH 4 with HCl, incubated at this pH for 2 min, followed by neutralization with NaOH. NaCl was added to a final concentration of 0.5 M and the sonicate was centrifuged at 10,000 x g for 60 min. The supernatant was loaded on an 85 x 153 mm column of heparin-Sepharose (Pharmacia, Piscataway, NJ) equilibrated with 0.5 M NaCl/3 mM EDTA/20 mM Tris, pH 7.5. The column was washed with the same buffer and was eluted with 2 M NaCl/3 mM EDTA/20 mM Tris, pH 7.5. The eluate was diluted with 3mM EDTA/20 mM Tris, pH 7.5 until the conductivity was 24 mmho and loaded on a second heparin-Sepharose column (16 x 190 mm). The column was washed with 0.7 M NaCl in 3 mM EDTA/20 mM Tris, pH 7.5, and was eluted with a 0.7 to 2 M NaCl gradient in the same buffer.

Fractions were assayed for protease-inducing activity and the active fractions were concentrated on a third heparin-Sepharose column (12 x 75 mm). This column was washed first with 0.8 M NaCl in 3 mM EDTA/20 mM Tris, pH 7.5 and then with 0.2 M NaCl in 0.1 M sodium phosphate, pH 6.0 and was eluted with 2 M NaCl in 0.1 M sodium phosphate, pH 6.0. The active fractions from the third heparin-Sepharose column were diluted with 20-times their volume of 0.1 M sodium phosphate, pH 6.0.

The solution was clarified by centrifugation at 10,000 x g for 30 min and was loaded on a 9 x 72 mm column of CM-Sephadex C-50 (Pharmacia) equilibrated with the same buffer. The column was sequentially eluted with 0.15, 0.5, and 2 M NaCl each in 0.1 M sodium phosphate, pH 6.0, and the fractions were assayed for protease-inducing activity.

The 0.5 M NaCl eluate of the CM-Sephadex column, which contained the protease-inducing activity, was concentrated on a 0.5 ml heparin-Sepharose column. This column was eluted with sequential 0.5 ml washes with 2 M NaCl in 60 mM sodium phosphate, pH 6.0. All activity was eluted in the first 1 ml. This

eluate was run on an FPLC Superose-12 column (Pharmacia) in 2 M
NaCl, 60 mM sodium phosphate, pH 6.0 with a flow rate of 0.5
ml/min.

An angiogenic factor within the scope of the present
claims was isolated from this eluate. This angiogenic factor is
referred to in part in this example as the "protein" or "pro-
teins."

  B. Characterization of Protein and Confirmation
    of Angiogenic Factor Properties

  1. NaDodSO$_4$-PAGE

NaDodSO$_4$ polyacrylamide gels with 3% stacking gels and
10 to 18% gradient resolving gels were prepared and run
according to the procedure of Laemmli as set forth in Nature
__277__: 680-685 (1970), specifically incorporated herein by refer-
ence. Proteins were detected with the silver stain procedure of
Wray __et al.__ as set forth in Anal. Biochem. __118__: 197-203 (1981).
The active fractions from this column contained a single band on
NaDodSO$_4$-PAGE with a molecular weight of 18,700.

  2. Protein determination

Protein concentrations were determined with the
Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using
bovine serum albumin as a standard. The sequence information
for this protein is set forth in Example 4.

  3. Mitogenic Properties –
    ^{125}I-iododeoxyuridine incorporation

Bovine capillary endothelial (BCE) cells were isolated
from the adrenal cortex of recently slaughtered yearling cattle
by the method of Folkman __et al.__ as reported in Proc. Natl. Acad.
Sci. USA __76__: 5217-5221 (1979), specifically incorporated herein
by reference. Cells were grown to confluence in alpha Minimal
Essential Medium (MEM) containing 10% (v/v) calf serum and sup-
plemented with medium conditioned by mouse sarcoma 180 cells as
described by Gross __et al.__ in J. Cell Biol. __95__: 974-981 (1982),
specifically incorporated herein by reference. When cultures
reached confluence, the medium was changed to MEM containing 5%
calf serum and no conditioning factors.

Confluent cultures of BCE cells were maintained in MEM
with 5% calf serum for 7 days. The medium was then replaced
with fresh MEM containing 5% calf serum and varying

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concentrations of purified placenta angiogenic factor. After 20 h, the medium was replaced with Dulbecco's Modified Eagle's medium containing 5% calf serum and 0.3 uCi/ml ^{125}I-iododeoxyuridine (2000 Ci/mmole, New England Nuclear, Boston, MA). After a 16 h incubation in labelling medium, labelling was terminated by washing the cells with cold phosphate buffered saline. Incorporation of ^{125}I-iododeoxyuridine into acid insoluble material was determined by incubating the cells in cold 5% trichloroacetic acid (TCA) for 30 min, washing twice with 5% TCA and distilled water. The TCA insoluble material was solubilized in 0.25 N NaOH and counted in a Packard 5210 gamma scintillation counter.

    4.   Migration assay

Migration assays were performed in 200 ul blind wells (Nucleopore, Pleasanton, CA) according to the method of Castellot as described in Proc. Natl. Acad. Sci. USA 79: 5597-5601, specifically incorporated herein by reference, using 5 um pore size polycarbonate PVP-free filters precoated with gelatin and fibronectin. Ten-fold serial dilutions of the purified protease-inducing factor in MEM containing 0.5% fetal calf serum were placed in the bottom wells. The filters were then inserted and 5 x 10^4 BCE cells in 200 ul MEM with 0.5% fetal calf serum were added to the upper wells. After a 4 h incubation at 37°C, the medium in the upper wells was removed and cells on the upper surfaces of the filters were gently scraped off with a cotton swab. Then the filters were removed, dried at room temperature, and stained with Wright-Giemsa stain (Baker Chemical Co., Phillipsburg, NJ). The total number of cells on the lower filter surface was counted under a light microscope (400X magnification).

    5.   Assays for the induction of PA and collagenase

Confluent cultures of BCE cells that had been maintained for at least two days in MEM containing 5% calf serum were changed to fresh MEM containing 5% calf serum and the substance to be tested. After incubation at 37°C for 24 h, the medium was collected from the cultures and was assayed for collagenase as described by Moscatelli et al. in Cell 20: 343-351 (1980), specifically incorporated herein by reference. All

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collagenase was in a latent form and was activated with trypsin to detect activity. The cell layers from these same cultures were washed twice with cold phosphate-buffered saline and were extracted with 0.5% (v/v) Triton X-100 in 0.1 M sodium phosphate, pH 8.1, and the cell extracts were assayed for PA activity as described by Gross et al., supra. Experiments have demonstrated that the amount of PA in cell extracts is proportional to the amount found in conditioned medium. One unit of protease-inducing activity was defined as the amount necessary to give half the maximal stimulation of PA and collagenase synthesis.

Example 2

    Purification of an angiogenic factor from Human Placental Tissue.

    The method of Example 1 was followed to obtain an eluate loaded onto the second heparin-Sepharose column. This column was washed with 0.95 M NaCl in 3mM EDTA/20mM Tris, pH 7.5, and was eluted with 2M NaCl in the same buffer.

    The 2M eluate was dialyzed against 0.2M NaCl/20mM MES, pH 6.0. The dialysate was clarified by centrifugation at 100,000xg for 60 min. and was loaded on a Mono-S column in a Fast Protein Liquid Chromatography (FPLC) system. The column was washed with 0.2M NaCl/20 mM MES, pH 6.0, and was eluted with a 0.2 to 2M NaCl gradient in 20mM MES, pH 6.0. Fractions were assayed for protease-inducing activity. The protease-inducing activity eluted at 0.45 to 0.6 M NaCl.

Example 3

    Purification of an Angiogenic Factor From Hepatoma Cells.

    All the purification steps, except the FPLC steps, were performed at 4°C. SK-Hep-1 cells (American Type Culture Collection (ATCC) Accession No. HTB 52) from confluent monolayers were scraped into cold PBS and pelleted by centrifugation at 400xg for 10 min. The cell pellet was suspended in 10 vol of PBS/0.5 M NaCl and sonicated for 3 min at 50 watts with a Branson Sonicator (Plainview, N.Y.). The extract was centrifuged (10,000xg, 1 h), and the supernatant was collected. The pellet was resuspended in 1 vol of PBS/0.5 M NaCl, sonicated and centrifuged.

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The two supernatants were pooled and passed through a
28 x 75 mm column of heparin-Sepharose (Pharmacia, Piscataway,
NJ) equilibrated with PBS/0.5 M NaCl. The column was washed
with 0.5 M NaCl/3 mM EDTA/ 100 mM Tris, pH 7.5 and eluted with a
0.5 to 2 M NaCl gradient in the same buffer. Fractions were
assayed for PA-inducing activity, and the active fractions were
pooled and diluted with 3 mM EDTA/ 20 mM Tris, pH 7.5 until the
conductivity was 20 mmho.

The active material was then passed through a second
heparin-Sepharose column (10 x 75 mm) equilibrated with 0.5 M
NaCl in 3 mM EDTA/ 20 mM Tris, pH 7.5. The column was washed
first with 0.5 M NaCl and then with 0.9 M NaCl in 3 mM EDTA/ 20
mM Tris, pH 7.5, and was eluted with a 0.9 to 2 M NaCl gradient
in the same buffer. The active fractions were concentrated on a
third heparin-Sepharose column (7 x 85 mm), which was washed
with 0.5 M NaCl and then eluted with 2 M NaCl, both in 20 mM
MES, pH 6.0. The active fractions from the third
heparin-Sepharose column were diluted 1:10 with 20 mM MES, pH
6.0, and the solution was clarified by centrifugation at
100,000xg for 1 h.

The same was then loaded on a Mono-S FPLC column
(Pharmacia) equilibrated with 0.2 M NaCl/ 20 mM MES, pH 6.0.
The column was washed with 0.2 M NaCl and elution was achieved
with a multilinear gradient of NaCl (0.2 to 2 M in 20 mM MES, pH
6.0). The fractions were assayed for PA-inducing activity and
the active fractions were pooled and purity was determined by
NaDod SO_4-PAGE.

Example 4

The Determination of the amino acid sequence of
placental angiogenesis factor (PAF) isolated from human
placenta.

A. LYS-C Peptides

Purified PAF in 20 mM MES, pH 6.0, 0.5 M NaCl was
obtained by the method of Example 2, above. The native protein
was subjected to digestion with endoproteinase Lys-C as follows:
A reaction mixture containing 2 nmoles of native protein in 350
ul of 20 mM MES, pH 6.0, 0.5M NaCl was adjusted to pH 8.7 by
addition of 15 ul of 2 M NH_4HCO_3, pH 9.0. 1.17 units of

endoproteinase Lys-C (Boehringer) was added and digestion was
carried out at 37°C for 7 hrs. and 30 min. 2-mercaptoethanol was
then added to a final concentration of 1% (v/v) and incubation
continued for 15 min. at 37°C. Trifluoroacetic acid (TFA) was
added to a final concentration of 0.1% (v/v) prior to the frac-
tionation of the digestion mixture by reverse phase high perfor-
mance liquid chromatography (HPLC) using a Synchrom RP-8 column.
The peptides were eluted from the column (flow rate 1.0 ml/min.)
with 0.1% TFA in water (5 min.) followed by a linear gradient of
acetonitrile made 0.1% in TFA (0 - 60% acetonitrile in 60 min.).
The elution of peptides was monitored at A_{215} and A_{280} and
appropriate fractions were collected manually.

A peptide eluting at 19% acetonitrile was sequenced by
automated Edman degradation and gave the following sequence:

(K)-N-G-G-F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K

In this and following sequences, an amino acid residue depicted
within parentheses is a residue which has not been unambiguously
identified.

A peptide eluting at 19.8% acetonitrile was sequenced
and gave the following result:

(K)-G-V-( )-A-N-( )-Y-L-(A)-M-K-(E)-D-G-

Another peptide from the same digest eluted at 17.5%
acetonitrile and gave the following amino acid sequence:

(K)-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K

A peptide that eluted at 26% acetonitrile was sub-
jected to automated Edman degradation and gave the following
amino acid sequence:

(K)-(C)-V-T-(D)-E-(C)-F-F-F-E-( )-L-E-S-N-N-Y-N-(T)-

Two additional peptides that eluted together at 16%
acetonitrile were collected as a mixture and then repurified
prior to sequencing. The collected peptide mixture was dried
under vacuum, then resuspended in 100 ul of 50 mM Tris-HCl, pH
8.5, 8 M urea. Twenty nmoles of dithiothreitol (DTT) was added
and the reduction of possible disulfide bonds was allowed to
proceed for 15 min. at 37°C. The peptides were then
carboxymethylated by addition of 60 nmoles at ^{3}H-iodoacetic acid
and the mixture was incubated for 20 min. in the dark at room
temperature. An additional 60 nmoles of DTT was added followed

by a 30 min. incubation at room temp. and the reaction mixture
was adjusted to 0.1% in TFA prior to refractionation by HPLC
using an Altex C-3 reverse phase column. The peptides were
eluted (flow rate 1 ml/min.) from the column with 0.1% TFA in
water (5 min.) followed by a linear gradient of acetonitrile
made 0.1% in TFA (0-60% acetonitrile in 120 min.).

 The peptide eluting at 13% acetonitrile was subjected
to automated Edman degradation and gave the following sequence:

  (K)-G-V-C-A-N-R-Y-L-A-M-K

B. SMP-Peptides

 Additional peptides were generated by digestion of the
native PAF protein with mouse submaxillary protease.

 A solution containing 2 nmoles of protein in 350 ul of
20 mM MES, pH 6.0, 0.5 M NaCl was adjusted to pH 8.0 by addition
of 25 ul of 1 M $NaHCO_3$, pH 9.0. Submaxillaris protease (3.6 ug)
was added and the digestion was allowed to proceed for 24 hrs.
at 37°C. 90 nmoles of DTT were added and the incubation at 37°C
continued for 30 min. Carboxymethylation of the peptides was
achieved by the addition of 360 nmoles of ^{3}H-iodoacetic acid and
incubation at room temperature for 20 min. in the dark. 360
nmoles of DTT were then added and the reaction mixture was
adjusted to 0.1% (v/v) in TFA prior to fractionation of the
peptide mixture by RP-8 HPLC as described above.

 A mixture of peptides eluting at 12% acetonitrile were
collected in one fraction, dried down and resuspended in 100 ul
of 50 mM Tris-HCL, pH 8.0, 8 M urea and then refractionated by
HPLC using an Altex C-3 column and the same elution schedule as
described above for the repurification of Lys-C peptides.

 Two peptides eluting at (a) 14.8% acetonitrile and (b)
15.3% acetonitrile were subjected to automated Edman degradation
and gave the following amino acid sequences:

  a) (R)-G-V-V-( )-I-K-G-V-C-A-N-

  b) (R)-L-V-C-K-N-G-G-F-F-

 Several peptides were generated by digestion of the
native PAF with <u>S. aureus</u> protease (V8). One mole of protein in
500 ul of 20 mM Tris-HCl, pH 7.5, 2M NaCl was desalted by HPLC
using an RP-8 reverse phase column. The salt free,
protein-containing fraction was dried down, resuspended in 50 ul

of 50 mM acetic acid, pH 4.0 and l ug of V8 protease was added.
Digestion was allowed to proceed for 18 hrs. at 37°C. Peptides
were then fractionated by HPLC using an RP-8 reverse phase col-
umn as described above.

A peptide eluting at 17% acetonitrile was subjected to
automated Edman degradation and gave the following amino acid
sequence:

(E)-K-S-( )-P-H-I-K-L-Q-L-( )-A-E

An additional peptide from the V8 digest that eluted
at 20% acetonitrile was also sequenced and gave the following
result:

(E)-( )-(G)-( )-L-L-A-( )-K-

A V8 peptide eluting at 21% acetonitrile was sequenced
with the following result:

(E)-S-N-N-Y-N-T-Y-R-(S)-

Ordering of all the amino acid sequences listed above
leads to a core sequence for the human basic fibroblast growth
factor as follows:

L-Y-C-K-N-G-G-F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-
S-( )-P-H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-A-N-
R-Y-L-A-M-K-( )-D-G-( )-L-L-A-( )-K-()-V-T-( )-E-( )-F-
F-F-E-( )-L-E-S-N-N-Y-N-T-Y-R-( )-

Additional peptides were isolated and subjected to
automated Edman degradation. These amino acid sequences are
outside the core amino acid sequence listed above.

A peptide eluting at 13% acetonitrile upon fractiona-
tion of the submaxillaris digest (see above) gave the following
amino acid sequence:

K-L-G-S-K-T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K

A LYS-C peptide eluting at 20% acetonitrile gave the
following amino acid sequence:

Y-( )-S-W-Y-V-( )-L-( )

Exmaple 5

Identification of Characteristics of the Angiogenic
Factor that Make it Suitable for Clinical Use as a Therapeutic
Agent.

We have demonstrated that the factor from placenta,
isolated by the method of Examples l or 2, and the factor iso-
lated from hepatoma cells have all three of the in vitro

properties predicted for an angiogenic factor. First, at con-
centrations in the range of 0.1 to 10 ng/ml, the molecule stimu-
lates the synthesis of PA and latent collagenase in BCE cells.
The PA can convert the zymogen plasminogen to active plasmin, a
protease of wide specificity. The plasmin can also convert la-
tent collagenase to active collagenase. Thus, under the influ-
ence of low concentrations of this factor, capillary endothelial
cells can generate at least two proteases which are able to
degrade most of the proteins in the surrounding tissues, which
would allow the cells to penetrate the tissues.

        The purified molecule stimulated PA and collagenase
synthesis in BCE cells in a dose-dependent manner (Fig. 3A).
All collagenase was in an inactive form. Collagenolytic activ-
ity was detected after trypsin treatment. Both PA and latent
collagenase are stimulated in parallel. Half maximal stimula-
tion occurred with a concentration of protease-inducing factor
of 1 ng/ml. The basal amount of PA and collagenase produced by
untreated cells varied from experiment to experiment, and, thus,
the extent of stimulation also varied. With very high concen-
trations of the protease-inducing factor, the stimulation of PA
synthesis was reduced as were the chemotactic and mitogenic
activities. Incubation of BCE cells for 24 hours with concen-
trations of the protease inducing factor that induced PA and
collagenase altered the morphology of the cells from their typi-
cal cobblestone appearance to a more elongated, spindle-shaped
appearance.

        Second, the factor is chemotactic for BCE cells. In
vivo, capillary endothelial cells, therefore, would be stimu-
lated to migrate toward the source of the factor. The addition
of the factor at concentrations between 0.001 and 0.1 ng/ml
stimulated BCE cell chemotaxis in blind well chambers. With
higher concentrations, stimulation of chemotaxis did not occur.
Increased cell movement from the upper chamber to the lower
chamber was detected only when the lower chamber contained a
higher concentration of factor than the upper chamber,
demonstrating that true chemotaxis was occurring. Chemokinesis
accounted for no more than 25% of the observed increased
motility.

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Third, the factor is mitogenic for BCE cells. Figure 3B demonstrates that addition of the protease-inducing factor to cultures of BCE cells stimulated the incorporation of ^{125}I-iododeoxyuridine into DNA in a dose-dependent manner. At higher concentrations of protease inducing factor, this stimulating effect was significantly reduced. Stimulation of ^{125}I-iododeoxyuridine incorporation was achieved with the same concentrations of factor which were able to induce PA and collagenase. We have previously determined that, with crude placenta sonicate, increased incorporation of ^{125}I-iododeoxyuridine into DNA correlates with other measurements of mitogenesis. Thus, this factor behaves as a bona fide endothelial cell mitogen. Thus, a single purified molecule seems to have the ability to induce PA and collagenase in BCE cells, to stimulate their replication, and to stimulate their motility.

Example 6

Angiogensis Activity

Using the method of Dunn et al., as published in Anat. Rec. 199: 33-42 (1981), for determining angiogensis, the angiogensis factor of Example 2, when placed on a chick chorioallantoic membrane, stimulated angiogensis in 81% of the eggs at a dose of 65ng.

Example 7

a) N-Terminal Amino Acid Sequence of PAF

Human placental angiogensis factor was purified as described previously (see Examples 1 and 2). The purified protein in 20 mM MES buffer, pH 6.0 and 500 mM NaCl was desalted by high performance liquid chromatography using an RP-8 reverse phase column. Two-hundred fifty to five hundred pmoles of desalted protein was applied to an ABI 470A gas-phase protein sequencer for automatic Edman degradation. The resultant PTH amino acids were identified by high performance liquid chromatography using a cyano reverse phase column.

These experiments resulted in the establishment of an N-terminal amino acid sequence for PAF as follows:

G-T-M-A-A-G-S-I-T-T-L-P-A-L-P-E

In addition, the N-terminal PAF amino acid sequence just described was also identified by automated Edman degradation of a Lys-C peptide that eluted at 23% acetonitrile in the chromatographic system described in Example 4, A.

     b)   C-Terminal Amino Acid Sequence of PAF

A C-terminal PAF peptide was isolated from the PAF Lys-C digest as described in Example 4. The peptide eluted at 22% acetonitrile. Automated Edman degradation of this peptide gave the following amino acid sequence:

     A-I-L-F-L-P-M-S-A-K-S

Combining sequence data from (i) Example 4; (ii) the N-terminal amino acid sequence of PAF; (iii) the C-terminal amino acid sequence of PAF; and (iv) cDNA, a complete amino acid sequence for PAF is as follows:

1.   PAF form 1

     G-T-M-A-A-G-S-I-T-T-L-P-A-L-P-E-D-G-G-S-

     G-A-F-P-P-G-H-F-K-D-P-K-R-L-Y-C-K-N-G-G-

     F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-S-D-P-

     H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-

     A-N-R-Y-L-A-M-K-E-D-G-R-L-L-A-S-K-C-V-T-

     D-E-C-F-F-F-E-R-L-E-S-N-N-Y-N-T-Y-R-S-R-

     K-Y-T-S-W-Y-V-A-L-K-R-T-G-Q-Y-K-L-G-S-K-

     T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K-S.

2.   PAF form 2:   N-terminally blocked PAF

From a number of experiments in which purified and intact PAF was subjected to automated Edman degradation, it became evident that a fraction of the applied protein (50-80%) is not degraded in the Edman procedure.

It is concluded that there exists a fraction of PAF protein molecules that are N-terminally blocked.

The nature of the blocking group is most clearly determined by the study of purified amino terminal PAF peptides. Amino terminal peptides from enzymatic degradations of PAF (see example 4) are identified by amino acid analysis. They may also be identified by paper electrophoresis or thin layer

chromatography followed by staining with the chlorine/o-tolidine
reagent as described by (Reindel, F. and Hoppe, W. (1954) in
Chem. Ber. 87, 1103-1107 specifically incorporated herein by
reference. (Blocked peptides are not detected with ninhydrin
(apart from a weak development of colour with the side-chains of
lysine residues) unless first hydrolyzed.)

        In addition, small, N-terminally blocked PAF peptides
may be isolated by chromatography of acidified thermolysin or
pepsin PAF digests on Dowex 50, X2 (H$^+$ form) as described by
Narita et al. (1975) in Protein Sequence Determination, pp.
30-103, Springer-Verlag, Berlin, Heidelberg, New York or by
chromatography on Sulphoethyl-Sephadex as described by Kluh, I.
(1979) in Coll. Czech. Chem. Comm. (Engl. Ed.) 44, 145-147, both
of which are specifically incorporated herein by reference.

        The structure of the short blocked peptides is deter-
mined by a variety of standard procedures and methods. For
example, see Allen, G. (1981) in Sequencing of Proteins and
Peptides; North Holland Publishing Company, Amsterdam, New York,
Oxford or references discussed therein, which are specifically
incorporated herein by reference. These procedures and methods
include digestion of the N-terminally blocked peptides with
carboxypeptidases, proglutamate aminopeptidase, hydrazinolysis,
mass spectrometry, nuclear magnetic resonance spectrometry, gas
chromatography, and fast-atom bombardment mass spectrometry as
described by Boissel et al. (1985) Proc. Natl. Acad. Sci. USA,
82, 8448-8452.

3. PAF form 3:   Truncated and Extended PAF

        It has been shown that bovine kidney fibroblast growth
factor (FGF) lacks a number of amino acids from the N-terminus
(Baird, A. et al. (1985); Regul. Pept; in press) (Gospodarowicz,
D. (1986) Meth. Enzymol., in press). The truncated fibroblast
growth factor retains its ability to bind to the FGF receptor,
as shown by Neufeld, G. and Gospodarowicz, D. (1985) in J. Biol.
Chem. 260, 13860-13868, indicating that the N-terminus of the
protein does not play a crucial role in the interaction of FGF
with its cell surface receptor. Therefore, is anticipated that
both truncated and extended forms of PAF will retain receptor
binding activity. The maximum N-terminal PAF deletion or

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extension that still allows for biological PAF activity remains
to be determined.

Example 8:   A cDNA Clone of PAF

          SK-HEP-1 cells were grown in Eagles Minimal Essential
Media supplemented with 10% fetal calf serum, non-essential
amino acids and Pen-Strep. RNA was isolated from cells using
the NP-40 lysis procedure as described by Maniatis et al. in Mo-
lecular Cloning: A Laboratory Manual (Cold Spring Harbor Labo-
ratory, New York, 1982, pg. 191-193), specifically incorporated
herein by reference. Poly (A)$^+$ mRNA was selected by oligo dT
chromatography (BRL) using the procedure of H. Aviv and P. Leder
described in Proc. Natl. Acad. Sci. USA 69, 1972, 1408 specifi-
cally incorporated herein by reference. Five ug of mRNA was
used to synthesize 8 ug of double stranded cDNA using oligo dT
primed 1st strand synthesis and RNase H-DNA polymerase mediated
2nd synthesis as described by Gubler and Hoffman in Gene, 25
(1983) 263-269, specifically incorporated herein by reference.
Amersham Reagents were used in this procedure. The following
reactions, unless otherwise stated, were done according to manu-
facturers specifications. This cDNA was blunt ended using 10
units T4 DNA Polymerase (Amersham). EcoRI sites were protected
with 400 units EcoRI methylase (New England Biolabs) and 100 mM
S-adenosyl methionine. An equal mass amount of EcoRI linkers
(New England Biolabs, 8 mer) were attached with 1 unit T4 DNA
ligase (Promega Biotec). Excess linkers were removed by
digesting with 200 units EcoRI (New England Biolabs) and 100 ng
of this cDNA was ligated into 1 ug of EcoRI-digested, alkaline
phosphatase-treated lambda gt-10 DNA (Vector Cloning Systems).
The DNA was the packaged in vitro (Vector Cloning Systems) and
when plated on E. coli, C600 HFLa yielded 8.2 x 10^5
recombinants.

Design of Oligonucleotide Probes

          Two mixed sequence oligonucleotide probes were used
for the isolation of the SK-HEP-1 cDNA clone. The probes con-
sist of pools of all possible DNA sequences for a given amino
acid sequence. Probes were made to selected amino acid se-
quences in the core PAF amino acid sequence described in this
application. Probe #5 was made to hybridize to DNA coding for

the amino acid sequence Ile-Lys-Gly-Val-Cys-Ala, and is a 17-mer
consisting of 192 sequences:

#5   Ile Lys Gly Val Cys Ala
       5'   ATZ AAN GGX GTX TGY GC   3'
            192-fold degenerate, 17 mer

Code X = A, T, G or C
     N = A or G
     Y = T or C
     Z = T, C or A

Probe #8 was made to hybridize to DNA coding for the amino acid
sequence Tyr-Cys-Lys-Asn-Gly-Gly-Phe, and is a 20 mer consisting
of 256 sequences:

#8   Tyr Cys Lys Asn Gly Gly Phe
       5'   TAY TGY AAN AAY GGX GGX TT   3'
            256-fold degenerate, 20 mer

Both probes were synthesized on an Applied Biosystems DNA syn-
thesizer. They were gel purified and radiolabeled with
$[\gamma-^{32}P]$ ATP (Amersham) using T4 polynucleotide kinase
(Pharmacia) to a specific activity of 4-6 x 10^6 cpm/pmol.

        Hybridization temperatures were chosen to be 2°C below
the calculated Tm for the most AT-rich member of each pool as
described by S.V. Suggs et al., (Developmental Biology Using
Purified Genes (eds. D.D. Brown and C.F. Fox), 683-693 (1982)
Academic Press, New York), specifically incorporated herein by
reference. The final wash was done at the calculated Tm for the
most AT-rich member of the pool (i.e., 2°C above the hy-
bridization temperature).

Screening

        The cDNA library was plated at a density of 50,000
plaques per 150 mm Luria-Bertoni agar plate with E. coli C600
HFLa cells and NZCYM top agarose (0.7%). Phage DNA was trans-
ferred to duplicate nitrocellulose filters (Schleicher and
Schuell, BA 85) and prepared for hybridization as described by
Benton and Davis in Science, 196: (1979) 180-182, specifically
incorporated herein by reference. The filters were
prehybridized at 48°C for 2 hours in a solution containing 6X
SSC (20X SSC is 3M NaCl, 0.3 M Sodium Citrate, pH 7.5), 2X
Denhardts Solution (100X Denhardts Solution is 2% Ficoll, 2%

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Polyvinyl pyrrolidone and 2% BSA), 0.1% SDS, 0.05% Sodium
Pyrophosphate and 100 mg/ml yeast tRNA. Probe #8 was added at
0.2 pmol/ml and allowed to hybridize for 16 hours. After hy-
bridization, the filters were washed as follows: 3 times for 15
minutes each in 6X SSC and 0.1% SDS at ambient temperature fol-
lowed by a final 8 minute wash at 50°C. The filters were then
dried and autoradiographed for 24 hours on Kodak XAR5 film and
one "lightening plus" intensifying screen at -70°C. Plaques
giving positive signals on duplicate filters were picked for pu-
rification. Those plaques were tested with probes #5 and #8 in
second round of purification and a plaque hybridizing to both
probes was chosen as the best candidate to code for the
angiogenesis factor.

     DNA was prepared from this phage by plate lysates and
formamide extraction as described by R.W. Davis, D. Botstein,
and J.R. Roth in Advanced Bacterial Genetics: A Manual for
Genetic Engineering (Cold Spring Harbor Laboratory, New York,
1980), specifically incorporated hereby in reference. An EcoRI
digest of this DNA released a 1.1 kb insert as sized in 1%
agarose gel. This insert was purified out of a 5% acrylamide
gel for subcloning as described by Maniatis et al., supra. at
173-178. The insert was ligated into EcoRI digested
Bacteriophage M13 mp 19 RF DNA and its sequence was determined
using the dideoxynucleotide method of Sanger et al. described in
J. Mol. Biol. 94, 441 (1975), specifically incorporated herein
by reference. Analysis of the sequence obtained showed an open
reading frame encoding the primary structure of PAF.

     Based on protein sequence data (see Example 7) and
published FGF information (Esch et al. (1985); Proc. Natl.
Acad. Sci. USA 82, 6507-6511) it appears that several active PAF
forms may be produced: Form 1 PAF (see also Example 7) may be
produced from this DNA by initiation of translation at some
point 5' to the sequence AGTMAA... and subsequent
post-translational cleavage of the AG bond by a process yet to
be established. In addition, a form 3 PAF may be produced by
initiation of translation at the MAA sequence, since this is a
consensus initiation site (M. Kozak, Microbiol. Rev. 1983, Vol.
47, 1-45) with optional proteolytic removal of the Methionine.

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Form 3 PAF may also be produced by initiation at other func-
tional initiation sites. These sites are readily discernable to
one of ordinary skill in the art, particularly in light of the
teachings contained herein. In addition, post-translational pro-
cessing of the initial translation product may then follow,
although such processing is not required. Form 2 PAF may be
produced from form 1 or form 3 of PAF by an as yet unknown pro-
cess leading to blockage of the free amino group at the
N-terminus.

Example 9:   Expression of PAF

The principle of the expression of PAF is as follows.
A 1.1 kb EcoRI fragment isolated from the lambda gt10 clone can
be subcloned into the plasmid pUC9. That fragment contains all
of the PAF coding sequence. Two smaller fragments from this
subclone are of utility in constructing expression systems. One
is a 367 bp AvaI to BamHI fragment which contains amino acid
residues 17 through 137 of the PAF coding sequence, counting the
GTMAA residues of the placental form 1 protein as 1-5. The
other is a 405 bp NcoI to BamHI fragment which contains amino
acid residues 4 through 137 of the PAF coding sequence. Syn-
thetic adaptors can then be attached to complete the coding se-
quence at both ends of these restriction fragments to provide
translational initiation, termination, or coupling sequences and
to supply the sequences necessary for attachment to the appro-
priate expression vector.

PAF isolated from human placenta contains a sequence
which starts with GTMAA. The cDNA clones isolated from SK-Hep-1
cells indicates that other forms of PAF may be synthesized
starting at least 100 amino acids upstream of the GTMAA se-
quence, or starting with MAA. The placental form was chosen for
expression in yeast (S. cerevisiae) and bacteria (E. coli). The
potential Sk-Hep-1 form and any other amino terminally truncated
form can be expressed by minor modifications of the procedures
described below that should be obvious to one skilled in the
art. They consist of altering the synthetic adaptors used to
attach the amino terminal end of the cDNA fragment (either the
NcoI site or the AvaI site) to the expression vectors. Alterna-
tively, plasmids expressing truncated forms can be constructed

from the GTMAA forms described below by oligonucleotide-directed
deletion mutagenesis (as described by M. Inouye, K. Nakamura, S.
Inouye, and Y. Masui in "Nucleic Research Future Development,"
K. Mizobuci, I. Watanabe, and J.D. Watson, eds., Academic Press,
New York, pp. 419-436, 1983, specifically incorporated herein by
reference).

Adaptors

The following adaptors were synthesized on an Applied
Biosystems DNA synthesizer and gel purified. The 5' ends were
phosphorylated with T4 polynucleotide kinase (Pharmacia). Pairs
of complementary oligonucleotides were annealed as follows to
form the double stranded adaptor. Equimolar amounts of each
oligonucleotide were added to a solution of 50mM NaCl, 10mM Tris
pH 7.5 and 1mM EDTA. This solution was heated in a boiling
water bath. The water bath was then removed from heat and
allowed to cool to ambient temperature over two hours. The fol-
lowing is a list and description of the adaptors used.

PAF Adaptors for attachment to the α-factor promoter of yeast

NH₃ terminal adaptors:

#1, GT Form

HindIII
site of
α-factor   #1A   5'   AGCTTGGATAAGAGAGGGAC   3'   To NcoI
                                                                            site of PAF
           #1B   3'   ACCTATTCTCTCCCTGGTAC   5'

#2, COOH terminal adaptor:

BamHI
site of
PAF   #2A   5'   GATCTAAAACAGGACCTGGGCAGAAAGCTATACTTTTTCTTCCAATGTCTGCTAAGAGCTGATAAGCC   3'   To SalI
                                                                                               site of
      #2B   3'   ATTTTGTCCTGGACCCGTCTTTCGATATGAAAAGAGGTTAGAGACGATTCTCGACTATTCGGAGCT   5'   α-factor

Adaptors for Expression in E. coli

For cytoplasmic production

#3   NH3 terminal adaptors for GT form

#3a   PvuI site   5'   CAAGGAGAAATAAATGGGACCATGGCAGCCCGGAGCATCACCACCGCCTGCCGCGCCTTGC   3'   AvaI site
#b   of Omp   3'   TAGTTCCTCTTTATTTACCCCTGGTACCGTCGGGCCTCGTAGTGGTGGCGGACGGGGCT   5'   of PGF

Omp   EcoRI to PvuI fragment

5'   AATTCGATATCTCGTTGGAGATATTCATTGACGTATTTTGGATGATAACGAGGCGCAAAAAATGAAAAAAGACAGCTATCGCGAT   3'
3'   GCTATAGAGCAACCTCTATAAGTACTGCATAAAACCTACTATTGCTCCGCGTTTTTTTACTTTTTTCTGTCGATAGCGC   5'

#4,   COOH terminal adaptor;

BamHI   #4A   5'   GATCTAAAACAGGACCTGGGCAGAAAGCTATACTTTTTCTTCCAATGTCTGCTAAGAGCTGACTGCA   3'   to PstI
site of   site of pCJ-1
PAF   #4B   3'   ATTTTGTCCTGGACCCGTCTTTCGATATGAAAAAGAAGGTTACAGACGATTCTCGACTG   5'

Adaptors for Secretion of PAF in E. coli using the Omp leader sequence

NH$_3$ terminal adaptors

#5   GT form:

#5a   5'   CCGGGACCATGCCAGCCGGGAGCATCACCACGCTGCCCGCCTTGC   3'   To AvaI site of PAF
#5b   3'   GGCCCTGGTACCGTCGGCCCTCGTAGTGGTGCGACGGGCGGAACGGGGCT   5'

Both of these adaptors will be ligated to the Omp leader described below.

5'   AATTCGATATCTCGTTGGAGATATTCATGACGTATTTTGGATGATGAACGAGGCGCAAAAAATGAAAAAAGACAGCTATCGGGATCCGCAGTTGC
3'   GCTATAGAGCAACCTCTATAAGTACTGCATAAAACCTACTATTGCTCCGCGGTTTTTTACTTTTTTCTGTCGATAGCGCTAGCGGTCACCG

5'   ACTGGCTGGTTCCCTACCGTAGCGCAGG   3'
3'   TGACCGACCAAGGCGATGGCATCGCGTCC   5'

#4, COOH terminal adaptor:

BamHI   #4A   5'   GATCTAAAAACAGAGACCTGGGCGCAGAAAGCTATACTTTTTCTTCCAATGTCTGCTAAGAGCTGACTGCA   3'   To PstI
site of   #4B   3'   ATTTTGTCCTGGACCCGCGTCTTTCGATATGAAAAAGAAGGTTACAGACGATTCTCGACTG   5'   site of pCJ-1
PAF

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                                                                    176                              206
GGG ACC ATG GCA GCC GGG AGC ATC ACC ACG CTG CCC TTG CCC GAG GAT GGC AGC
Gly Thr Met Ala Ala Gly Ser Ile Thr Thr Leu Pro Ala Leu Pro Glu Asp Gly Gly Ser

                                                                    236                              266
GGC GCC TTC CCG CCC GGC CAC TTC AAG GAC CCC AAG CGG CTG TAC TGC AAA AAC GGG GGC
Gly Ala Phe Pro Pro Gly His Phe Lys Asp Pro Lys Arg Leu Tyr Cys Lys Asn Gly Gly

                                                                    296                              326
TTC TTC CTG CGC ATC CAC CCC GAC GGC CGA GTT GAC GGG GTC CGG GAG AAG AGC GAC CCT
Phe Phe Leu Arg Ile His Pro Asp Gly Arg Val Asp Gly Val Arg Glu Lys Ser Asp Pro

                                                                    356                              386
CAC ATC AAG CTA CAA CTT CAA GCA GAG GAG AGA GTT GTG TCT ATC AAA GGA GTG TGT
His Ile Lys Leu Gln Leu Gln Ala Glu Glu Arg Gly Val Val Ser Ile Lys Gly Val Cys

                                                                    416                              446
GCT AAC CGT TAC CTG ATG AAG GAA GAT GGA TTA CTG GCT TCT AAA TGT GTT ACG
Ala Asn Arg Tyr Leu Ala Met Lys Glu Asp Gly Arg Leu Leu Ala Ser Lys Cys Val Thr

                                                                    476                              506
GAT GAG TGT TTC TTT GAA CGA TTG GAA TCT AAT AAC TAC AAT ACT TAC CGG TCA AGG
Asp Glu Cys Phe Phe Glu Arg Leu Glu Ser Asn Asn Tyr Asn Thr Tyr Arg Ser Arg

                                                                    536                              566
AAA TAC ACC AGT TGG TAT GTG GCA CTG AAA CGA ACT GGG CAG TAT AAA CTT GGA TCC AAA
Lys Tyr Thr Ser Trp Tyr Val Ala Leu Lys Arg Thr Gly Gln Tyr Lys Leu Gly Ser Lys

                                                                    596
ACA GGA CCT GGG CAG AAA GCT ATA CTT CTT CCA ATG TCT GCT AAG AGC TGA
Thr Gly Pro Gly Gln Lys Ala Ile Leu Leu Pro Met Ser Ala Lys Ser End
```

<u>Example 9</u>:   <u>Construction of Yeast Expression Plasmids</u>

      A plasmid, pGS185, derived from pUC 8 but lacking the restriction sites in the polylinker from the <u>Hind</u> III site to the <u>SmaI</u> site, was constructed by digesting pUC 8 with <u>Hind</u> III, ligated it to a <u>Hind</u> III/<u>SmaI</u> adaptor (Amersham, Cat. No. DA1006) which does not reconstruct the <u>Hind</u> III site, digesting with <u>SmaI</u> and ligating in dilute solution (1 ng/ml) followed by transformation of <u>E. coli</u> JM 83. The correct plasmid, was identified by digesting plasmid DNA isolated from transformants with <u>EcoRI</u>, <u>SmaI</u> or <u>Hind</u> III. A transformant containing a plasmid that lacked the <u>Hind</u> III site but contained the <u>EcoRI</u> site and <u>SmaI</u> site was identified in this manner.

      An <u>EcoRI</u> fragment containing yeast MF 1 gene was purified by gel electrophoresis from the plasmid pCY17 as described by J. Kurgan and I. Herskowitz in Cell 30:933 (1982) and ligated into <u>EcoRI</u> cut pGS185. This ligation mixture was used to transform <u>E. coli</u> HB101, selecting for ampicillin resistance. Plasmid DNA was isolated from transformants and the presence of the correct insert confirmed by digests of the DNA with <u>EcoRI</u>. This is plasmid pGS285.

      Plasmid pGS285 was digested to completion with <u>Hind</u> III and religated under dilute conditions (1 ng/ml) to eliminate three of the four internal <u>Hind</u> III sites in the MF 1 gene as noted by Kurjan and Herskowitz, <u>ibid</u>. The correct construct was selected as described above. This is plasmid pGS385.

      For site-directed mutagenesis, the MF 1 gene was removed from pGS385 by digestion with <u>EcoRI</u>, gel purified (1.5 Kb) and ligated to <u>EcoRI</u> digested M13 mp18 RF. The ligation mixture was used to transform <u>E. coli</u> 71-18 and clones containing the MF 1 gene in the correct orientation were identified by hybridization to the [^{32}P] labeled MF 1 gene. The MF 1 sequence was changed from GTA TCT TTG GAT AAA AGA to GTA AGC TTG GAT AAA AGA using standard site directed <u>in vitro</u> mutagenesis methods described by Zoller and Smith (Methods in Enzymology, Vol. 100, 1983, Academic Press, Inc., p. 468). The sequence of the mutant -factor gene, MF -H, was confirmed by dideoxy sequencing.

      The MF -H gene was removed by digesting the RF form of the M13 mp18 clone with <u>EcoRI</u>, gel-purifying the resulting 1.5 Kb <u>EcoRI</u> fragment by acrylamide gel electrophoresis and ligating it to <u>EcoRI</u> cut pGS185. The resulting ligation mixture was used to transform <u>E. coli</u> HB101 and colonies containing the plasmids with the MF -H gene were identified by hybridization with ^{32}P labeled 1.5 Kb <u>EcoRI</u> fragment containing the MF -H gene. This plasmid is designated pGS286.

The PAF gene was inserted into pGS286 as follows. Adaptors #1
and #2 were ligated to the PAF NcoI/BamHI fragment. The ligation mixture
was electrophoresed on a polyacrylamide gel and PAF DNA with the attached
adaptors identified by an increase in MW. This correctly adapted DNA frag-
ment was eluted from the gel, and was ligated to Hind III/SalI digested
pGS286. E. coli HB101 was transformed with the ligation mixture and
ampicillin resistant colonies were selected. Transformants containing
plasmids with the correct insert were identified by hybridization with
adaptor 1A and 2A, radio labelled by incubation with [γ - ^{32}P]ATP and T4
polynucleotide kinase. A plasmid constructed and isolated in this manner
has been designated pGS286-PAF. This plasmid contains the MF H gene
fused, in frame, to the PAF gene at the Hind III site in the "pre-pro" re-
gion of the MF H gene. Such constructs, when placed in yeast, have been
demonstrated to direct the synthesis, processing and secretion of
heterologous proteins as shown by A.J. Brake et al., 1981 (PNAS (USA)
81:4642).

        The EcoRI fragment containing the fusion of the MF H gene and
PAF is pGS286-PAF. This fragment was isolated by digestion with EcoRI and
polyacrylamide gel electrophoresis. It was made blunt ended with T4 DNA
polymerase and PstI adaptors (Pharmacia) were attached with T4 DNA ligase.
The fragment was then ligated into PstI digested vector pCl/1 (A.J. Brake et
al. 1981) (PNAS, (USA) 81:4642) and E. coli HB101 transformed with the liga-
tion mix and TET^r colonies were selected. Correct constructs were identi-
fied by hybridization to the PAF gene. This plasmid was introduced into S.
cerevisiae DBY 746 (Yeast Genetic Stock Center, Berkeley, CA), with the two
micron DNA plasmid deleted as described by Toh-E and Wickner (Journal of
Bacteriology, 145, 1981, 1421-1424), by standard transformation protocols.
Transformants expressing PAF were selected by their reactivity with affinity
purified anti PAF IgG.

Example 10: Periplasmic Secretion in E. coli

        To regulate the expression of PAF in a form suitable for export
to the periplasm of E. coli, the following regulatory elements were used: a
tac promoter on plasmid pCJ-1 for initiation of transcription at high lev-
els; a lac operator on plasmid pCJ-1 for transcription regulation; a lac re-
pressor (lac I), encoded on the chromosome of E. coli strain JM107. To
facilitate periplasmic export of PAF, DNA coding for the Omp A leader
peptide was attached to the DNA coding for PAF in such a way that the
C-terminal Ala of this peptide will be fused to the N-terminal Gly of PAF

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form 1 in such a way that the Ala-Gly bond of the initial product will be
cleaved by the E. coli leader peptidase to yield the mature PAF.

The E. coli secretion vector was constructed as follows.
Adaptors #5 and #4 were ligated to the PAF AvaI/BamHI fragment. DNA of the
correct size was eluted from a polyacrylamide gel and ligated to the Omp A
leader DNA and EcoRI/PstI digested M13 mp19 RF. E. coli JM-107 were trans-
formed with the ligation mix. Transformants containing the PAF gene were
detected by restriction mapping and the sequence of the construct was con-
firmed by dideoxy sequencing. The EcoRI/PstI fragment containing the PAF
gene was isolated from the RF DNA by restriction with EcoRI and PstI and
elution from a polyacrylamide gel. This was ligated into EcoRI/PstI
digested pCJ-1 and E. coli JM107 were transformed with the ligation mixture.
Colonies producing PAF were selected by growth on Tet plates and
immunoscreening with affinity purified anti-PAF IgG.

Example 11: Cytoplasmic Expression in E. coli

To regulate the expression of PAF in a form such that the PAF
remains in the E. coli cytoplasm, the following operational elements were
used: the tac promoter on plasmid pCJ-1; the lac operator of the plasmid
pCJ-1 and the lac repressor (lac I^q) on the chromosome of E. coli strain
JM107; a consensus Shine-Dalgarno sequence; and, to initiate a high level of
translation, a fragment of the Omp A leader peptide to be used as a transla-
tional coupler. The translational coupling sequence comprises the DNA
coding for the translation initiation region of the Omp A gene, the first
eight amino acids of the Omp A leader peptide, the consensus Shine-Dalgarno
sequence described above and a translational terminator. The translational
coupling sequence is to be inserted between the lac operator and the trans-
lation initiation site of the PAF gene, overlapping the latter. (The fea-
tures of the translational coupler are incorporated on the DNA sequence
shown with the adaptors for secreted expression in E. coli.)

The PAF gene was incorporated into the pCJ-1 plasmid with the
translational coupler as follows. Adaptor #3 and the Omp A translational
coupler were attached to the PAF AvaI/PstI fragment from the M13 mp19 con-
struct described in Example 10. This fragment was purified from a poly-
acrylamide gel. This fragment was then ligated into EcoRI/PstI digested M13
mp19 RF and the ligation mix used to transform E. coli JM 107 cells.
Plaques containing the PAF gene fusion were chosen by restriction mapping.
The sequence of the construct was then confirmed by dideoxy sequencing. The
EcoRI/PstI fragment containing the PAF gene fusion was eluted from a

polyacrylamide gel and ligated into EcoRI/PstI digested pCJ-1 and E. coli
JM107 cells were transformed with the ligation mix. Colonies showing
tetracycline resistance were selected and PAF production was confirmed by
immunoscreening with affinity purified anti-PAF IgG.

It will be apparent to those skilled in the art that various
modifications and variations can be made to the processes and products of
the present invention. Thus, it is intended that the present invention
cover these modifications and variations of this invention provided they
came within the scope of the appended claims and their equivalents.

Example 12: Cytoplasmic Expression in E. coli

The M13 and mp19 secretion construct described in Example 10 was
digested with NruI and NcoI and the large fragment was eluted from a gel.
Adaptor #6 was then ligated into the NruI/NcoI cut DNA. E. coli strain
JM107 was transformed with the ligation mix. Plaques containing the PAF
gene fusion were confirmed by dideoxy sequencing. The EcoRI/PstI fragment
containing the PAF gene fusion was eluted from a polyacrylamide gel and
ligated into EcoRI/PstI digested pCJ-1 and E. coli JM107 cells were trans-
formed with the ligation mix. Colonies showing tetracycline resistance were
selected and PAF production was confirmed by immunoscreening with affinity
purified anti-PAF IgG.

Adaptor #6

#6a NruI site 5' CGATCAAGGAGAAATAAATGGGGAC   3' To NcoI

#6B OF Amp   3' GCTAGTTCCTCTTTATTTACCCCTGGTAC 5' site of PAF

Example 13: Identification of mRNA

Total RNA was isolated from the following cell lines: SK HEP-1
human hepatoma cells, human embryonic lung (HEL) cells, RPMI 7272 human
melanoma cells and mouse sarcoma 180 cells. Poly A plus minus messenger RNA
(mRNA) was then isolated using oligo (DT) cellulose chromatography. Fifteen
micrograms of poly A plus minus RNA from each of the above cell lines was
separated by electrophoresis on a 1.25 percent agarose/formaldehyde gel.
These RNAs were transferred to a zeta-probe membrane. A cDNA probe to human
basic FGF from SK HEP-1 human hepatome cells (FGF15, the EcoRI insert) was
labeled with ^{32}P-DCTP by the NICK translation procedure to a specific activ-
ity of 8.64 X 10^8 cpm/ug. Hybridization of the ^{32}P-BFGF cDNA probe to the
transferred RNA was carried out in 50 percent formamide, 6X SSC, 2X
Denhardt's solution, 1 percent SDS, 0.05 percent sodium pyrophosphate and
175 ug/ml tRNA for 16 hours at 42°C. The zeta-probe membrane was then
washed in 1 liter each of 0.5X SSC/1 percent SDS, 0.2X SSC/1 percent SDS,

0.1X SSC/1 percent SDS for 15 minutes at 65°C and in 0.1X SSC/1 percent SDS
for 15 minutes at 70°C. The membrane was then dried and autoradiography
carried out for 1 and 3 days at -80°C. SK HEP-1 cells, HEL cells and RPM1
7272 cells each contained four species of RNA having sizes of 8.0 KB, 4.3
KB, 2.3 KB and 1.0 KB which hybridized to the cDNA probe described above.
The cDNA probe did not hybridize to any RNA species from mouse sarcoma 180
cells.

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CLAIMS

1. An angiogenic factor comprising a purified, human or synthetic single-chain polypeptide protein exhibiting substantial homology to the native angiogenic factor isolatable from human placental tissue, wherein said angiogenic factor has at least one active site possessing an activity selected from the group consisting of mitogenic activity, chemotactic activity, the ability to stimulate protease synthesis, and combinations thereof.

2. An angiogenic factor comprising a purified, single-polypeptide-chain protein having at least one active site possessing mitogenic and chemotactic activity and the ability to stimulate protease synthesis, said protein exhibiting substantial homology to the native angiogenic factor isolatable from human placental tissues.

3. The angiogenic factor of claim 1 wherein said angiogenic factor has at least one active site possessing chemotactic activity and which has the ability to stimulate protease synthesis.

4. The angiogenic factor of claim 1 wherein said angiogenic factor has at least one active site possessing mitogenic activity and the ability to stimulate protease synthesis.

5. The angiogenic factor of claim 1 wherein said angiogenic factor is isolated from human placenta in a substantially purified form.

6. An angiogenic factor protein comprising a purified, single-polypeptide-chain protein having at least one active site possessing mitotic and chemotactic activity and the ability to stimulate protease synthesis, wherein said protein comprises in part the amino acid sequences:

L-Y-C-K-N-G-G-F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-
S-()-P-H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-A-N-
R-Y-L-A-M-K-()-D-G-()-L-L-A-()-K-()-V-T-()-E-()-F-
F-F-E-()-L-E-S-N-N-Y-N-T-Y-R-()-;

K-L-G-S-K-T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K; and

Y-()-S-W-Y-V-( )-L-( ).

7.   An angiogenic factor protein comprising a purified, single-polypeptide-chain protein having at least one active site possessing mitotic and chemotactic activity and the ability to stimulate protease synthesis, wherein said protein comprises in part the amino acid sequence:

G-T-M-A-A-G-S-I-T-T-L-P-A-L-P-E-D-G-G-S-

G-A-F-P-P-G-H-F-K-D-P-K-R-L-Y-C-K-N-G-G-

F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-S-D-P-

H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-

A-N-R-Y-L-A-M-K-E-D-G-R-L-L-A-S-K-C-V-T-

D-E-C-F-F-F-E-R-L-E-S-N-N Y-N-T-Y-R-S-R-

K-Y-T-S-W-Y-V-A-L-K-R-T-G-Q-Y-K-L-G-S-K-

T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K-S.

8.   An angiogenic factor protein wherein the amino acid sequence of the protein differs from the sequence in claim 7 by one amino acid residue.

9.   An angiogenic factor protein wherein the amino acid sequence of the protein differs from the sequence in claim 7 by two amino acid residues.

10.   The angiogenic factor protein of claim 7 wherein said protein is N-terminally blocked.

11.   A method for obtaining an angiogenic factor in pure form from tissues comprising:

(a)   collecting tissue capable of producing the angiogenic factor;

(b)   isolating the angiogenic factor from the tissue by fractionating the proteinaceous material in the tissue;

(c)   identifying the fractions which possess angiogenic factor activity; and

(d)   concentrating the fractions exhibiting angiogenic factor activity wherein the angiogenic factor comprises single-chain polypeptide protein exhibiting substantial homology to the native angiogenic factor isolatable from human placental tissue wherein said angiogenic factor has at least one active site possessing an activity selected from the group consisting of mitogenic activity, chemotactic activity, ability to stimulate protease synthesis, and combinations thereof.

12.   The method of claim 11 wherein the tissue capable of producing the angiogenic factor is human placental tissue.

SUBSTITUTE SHEET

13. A method for the production of angiogenic factor wherein
said angiogenic factor comprises a single-chain polypeptide exhibiting sub-
stantial homology to the native angiogenic factor isolatable from human
placental tissue wherein said angiogenic factor has at least one active site
possessing an activity selected from the group consisting of mitogenic
activity, chemotactic activity, the ability to stimulate protease synthesis,
and combinations thereof, comprising:

    (a) isolating a DNA sequence encoding the angiogenic
        factor;

    (b) inserting the DNA sequence into a vector capable of
        expression in a host microorganism;

    (c) transforming the vector containing the DNA sequence
        into a host microorganism capable of expressing the
        angiogenic factor;

    (d) expressing the angiogenic factor from the trans-
        formed microorganism; and

    (e)· in either order, isolating and purifying the
        expressed angiogenic factor.

14. The method of claim 13 wherein the host microorganism is
transformed with pGs286-PAF.

15. The method of claim 13 wherein the DNA sequence encodes a
protein containing the amion acid sequence:

L-Y-C-K-N-G-G-F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-
S-()-P-H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-A-N-
R-Y-L-A-M-K-()-D-G-()-L-L-A-()-K-()-V-T-()-E-()-F-
F-F-E-()-L-E-S-N-N-Y-N-T-Y-R-()-;

K-L-G-S-K-T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K; and

Y-( )-S-W-Y-V-( )-L-( ).

16. The method of claim 13 wherein the DNA sequence encodes a
protein containing the amion acid sequence:

G-T-M-A-A-G-S-I-T-T-L-P-A-L-P-E-D-G-G-S-

G-A-F-P-P-G-H-F-K-D-P-K-R-L-Y-C-K-N-G-G-

F-F-L-R-I-H-P-D-G-R-V-D-G-V-R-E-K-S-D-P-

H-I-K-L-Q-L-Q-A-E-E-R-G-V-V-S-I-K-G-V-C-

A-N-R-Y-L-A-M-K-E-D-G-R-L-L-A-S-K-C-V-T-

D-E-C-F-F-F-E-R-L-E-S-N-N-Y-N-T-Y-R-S-R-

K-Y-T-S-W-Y-V-A-L-K-R-T-G-Q-Y-K-L-G-S-K-

T-G-P-G-Q-K-A-I-L-F-L-P-M-S-A-K-S.

17. The method of claim 13 wherein the host organism is <u>E.</u>
<u>coli</u>.

18. The method of claim 13 wherein the host organism is a
yeast.

INTERNATIONAL SEARCH REPORT

International Application No   PCT/US86/02669

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.CL.   530/324,350; 435/68,71,172.3

INT. CL. -4- CO7K 7/10, 13/00; C12P 21/00, 21/04; C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
US	530/324,350; 435/68,71,172.3

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
P,X	Biological Abstract, Vol. 81, Issued 01 May 1986, abstract no. 79778, (Mignatti)	1-12
X,	Biological Abstract, Vol. 77, Issued February 1984, abstract no. 17462, (Burgos)	1-12
X,	FEBS Letter, Vol. 184, no. 2, Issued May 1985, pages 255-258, (Buki)	1-12
P,X,	Proceeding National Academy Science Vol. 83, pages 2091-2095, Issued April 1986, (Moscatelli)	1-18
X,	US,A, Patent No. 4,225,670, Published 30 September 1980, (Tolbert)	1-12
X,	US,A, Patent No. 4,529,590, Published 16, July 1985, (LeVeen)	1-12

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ³	Date of Mailing of this International Search Report ³
24 February 1987	1 1 MAR 1987

International Searching Authority ¹	Signature of Authorized Officer ²⁰
ISA/US	

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

   I.   Claims 1-12 drawn to angiogenic factor and method for obtaining such from tissue classified in Class 530 Subclass 324 and 350.

1.☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

  ☐ The additional search fees were accompanied by applicant's protest.

  ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (October 1981)

ATTACHMENT TO FORM PCT/ISA/210, PART VI.

II. Claims 13-18 drawn to methods of formation of
the angiogenic factor by generic engineering
classified in Class 435 Subclass71.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
X,	US,A, Patent No. 4,209,587, Published 24 June 1980, (Tolbert)	1-18
X,	US,A, Patent No. 4,210,718, Published 1 July 1980, (Tolbert)	1-18
X,	US,A, Patent No. 4,210,719, Published 1 July 1980, (Tolber)	1-18
X,	US,A, Patnet No. 4,217,412, Published 12 August 1980, (Tolbert)	1-18
X,	US,A, Patent No. 4,273,871, Published 16 June 1981 (Tolbert)	1-18
X,	US, A, Patent No. 4,229,532, Published 21 October 1980, Tolbert)	1-18
X,	US,A, Patent No. 4,268,629, Published 19 May, 1981 (Tolbert)	1-18
X,	US,A, Patent No. 4,229,531, Published 21 October 1980 (Tolbert)	1-18
P,X,	Chemical Abstract, Issued 1986, abstract no. 200508z, Vol. 104, (Moscatelli)	1-18
P,X,	Chemical Abstract, Vol. 104, Issued 1986, abstract no. 82706n, (Mignatti)	1-18